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13. ABSTRACT (Maximum 200 Words)

Alteration of the Fragile Histidine Triad (*FHIT*) gene, encompassing the FRA3B fragile site at chromosome 3p14.2, especially reduction or deletion of its expression, is involved in many breast cancers. Little is known about the biological function of the Fhit in breast cancer progression. The major goal of this proposal is to test the hypothesis that the over-activated ATR pathway in irradiated $Fhit^{-/-}$ cells promote the homologous recombination repair (HRR) of DNA DSBs, resulting in more $Fhit^{-/-}$ cells surviving with deletion or translocations at fragile sites (malignant feature). In the passed year with this grant support, we found that the over-activated ATR pathway regulated checkpoint contributes the radioresistance of $Fhit^{-/-}$ cells and ATR is linked to HRR but not non-homologue end joining repair. These results will provide theory guidance for improving clinical treatment of breast cancers in which Fhit is deleted or reduced by combining traditional therapy with blocking the ATR pathway.

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INTRODUCTION:

Alteration of the Fragile Histidine Triad (*FHIT*) gene, encompassing the FRA3B fragile site at chromosome 3p14.2 (1), especially reduction or deletion of its expression, is involved in many solid tumors including breast cancer. It is reported that a complete loss or a significant reduction of Fhit expression was observed in 72% of breast carcinomas examined and a correlation between a decrease or an absence of Fhit protein expression and high proliferation and large tumor size of breast cancer was identified (2). Little is known about the biochemical and biological function of the Fhit in breast cancer progression. DNA double strand break (DSB) that may occur spontaneously and can be induced by DNA damage reagents such as ionizing radiation (IR) is one of the frequent reasons for promoting breast tumor genesis and development. The purpose of this proposal is to test our hypothesize that the over-activated ATR pathway in irradiated Fhit$^{-/-}$ cells promote the homologous recombination repair (HRR) of DNA DSBs, resulting in more Fhit$^{-/-}$ cells surviving with deletion or translocations at fragile sites (malignant feature).

BODY:

Two major pathways repairing DNA DSB exist in mammalian cells: non-homologous rejoining (NHEJ) and homologous recombination repair (HRR). By studying Fhit knockout cell lines obtained from Dr. Huebner's laboratory (3), we observed that Fhit$^{-/-}$ cells are more resistant to IR-induced killing than Fhit$^{+/+}$ cells. The radioresistant phenotype of Fhit$^{-/-}$ cells is associated with stronger S and G2 checkpoint responses cells (Fig. 1) regulated by an over-activated ATR pathway (Fig. 2-4) (4). In addition, we found that ATR protects cell from IR-induced killing is linked to HRR but not NHEJ (5). The results are briefly described in the following paragraphs.

MATERIALS AND METHODS

Cell lines, chemical treatment and irradiation. Fhit$^{+/+}$ and Fhit$^{-/-}$ epithelial cells from mouse kidney, generated as described earlier (3), were immortalized by tissue culture passaging. These cells were adapted to growth in DMEM supplemented with 10% iron-supplemented calf serum (Sigma-Aldrich Co. USA) at 37°C in an atmosphere of 5% CO_2 and 95% air. Caffeine (Sigma-Aldrich Co.), or UCN-01 (National Cancer Institute, USA) was added to the culture 30 min before the cells were exposed to X-rays (310 kV, 10 mA, 2-mm Al filter) and was kept in the culture until the cells were collected.

DNA synthesis. The S phase (S) checkpoint is detected by measuring DNA synthesis, which is similar to that described previously (6). Briefly, 1×10^5 cells from a growing culture were seeded in 60-mm tissue culture dishes with 3 ml of medium containing 10 nCi of [^{14}C]-thymidine and allowed to grow for more than one doubling time. This [^{14}C] pre-labeling provides an internal control for cell number by allowing normalization for total DNA content of samples.

Before irradiation, the cell cultures were changed with pre-warmed medium (washed off [^{14}C]-thymidine) containing either caffeine or UCN-01 for 30 min. Cells were exposed to X-rays (310 kV, 10 mA, 2-mm Al filter) at room temperature and returned to

37°C. The chemicals were kept in the culture until cells were harvested. [^{3}H]-thymidine at 0.5 μCi was added to the culture for 30 min at 3 h after IR and the cells were then collected. The rate of DNA synthesis for each sample was calculated as ^{3}H dpm/^{14}C dpm and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time-point, as described previously (6).

Flow cytometry assay. The G2 checkpoint is detected by flow cytometry measurement. As described previously (7), Fhit cells were collected at required times and fixed in 70% ethanol. Cells were washed with PBS and stained with a solution containing 62 μg/ml RNase A, 40 μg/ml propidium iodide and 0.1% Triton X-100 in phosphate-buffered saline at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite, USA).

Kinase activity and western blot. Nuclear extracts were prepared by using the NE-PER™ kit (PIERCE, USA) according to the manufacturer's instructions. The fractions of chromatin-bound extract were prepared as described previously (8). Briefly, cells were collected and washed in cold phosphate-buffered saline. Proteins were then extracted with cold 0.1% Triton X-100 in CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfony fluoride) for 20 min at 4°C. The chromatin-bound fraction was then pelleted by low-speed centrifugation at 3,000 rpm for 5 min at 4°C. The supernatant was named fraction 1. These pellets were then re-extracted by incubating in the same CSK buffer and were collected by centrifugation at 3,000 rpm for 10 min at 4°C. This supernatant was named fraction 2. The final pellet fraction (containing chromatin-bound proteins) was solubilized in radioimmunopreciptation assay (RIPA) buffer (150 mM NaCl, 40 mM MOPS, pH 7.2, 1 mM EDTA, 1% NP40, 1% Sodium deoxycholate, 0.1% SDS) and was named fraction 3. For ATR kinase assay, 500 μg of fraction 3 was then mixed with 2 μg of ATR antibody (sc-1887, Santa Cruz Biotechnology, Inc. USA) in the presence of 20 μl of a 50% (v/v) protein G-Sepharose slurry (RepliGen, USA) in 500 μl of Buffer A (0.5% NP-40, 1 mM Na_3VO_4, 5 mM NaF, 0.2 mM PMSF in PBS buffer). For CHK1 kinase assay, 250 μg of nuclear extract was then mixed with 1 μg of CHK1 antibody (sc-7898, Santa Cruz Biotechnology, Inc.) in the presence of 10 μl of a 50% (v/v) protein A-Sepharose slurry in 250 μl of Buffer A. These mixtures were gently rotated overnight at 4°C. Immune complexes were washed twice with Buffer A, then twice with Buffer B (10 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT). The ATR kinase immunoprecipitate supplemented with 1 μg of PHAS-1 (Stratagene, USA) and the CHK1 kinase immunoprecipitate supplemented with 1 μg of purified GST-$CDC25C_{200-256}$ (8) were incubated at 30°C for 30 min in 20 μl Buffer B containing 10 μCi (γ-^{32}P) ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by measuring the incorporation of ^{32}P into PHAS-1 protein (ATR kinase) or into $CDC25C_{200-256}$ (CHK1 kinase) with the PhosphoImager. Antibodies against ATR (sc-1887, Santa Cruz Biotechnology, Inc. USA), CHK1 (sc-8404, Santa Cruz Biotechnology, Inc.) and CDC25A (sc-7389, Santa Cruz Biotechnology, Inc.) were used in the Western Blot.

Colony-forming assay. Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (7).

Transfection of Atr and Chk1 siRNA. The *Atr* siRNA was designed to specifically target the sequences of the conserved region between rat and mouse *Atr* mRNA (5'-AAGACAGATTCTCTGCCAGTT-3'). The *Chk1* siRNA was designed to specifically target the sequences of the conserved region among human, rat and mouse *Chk1* mRNA (5'-AAGTTCAACTTGCTGTGAATA-3'). The siRNAs were synthesized by Dharmacon, Inc. Scrambled duplex RNAs (Dharmacon, Inc. USA) were used in the control transfection. The RNAs were delivered to the cells by OLIGOFECTAMINE™ (Invitrogen Corp. USA), according to the manufacturer's instructions. The cells were analyzed at 36 h posttransfection.

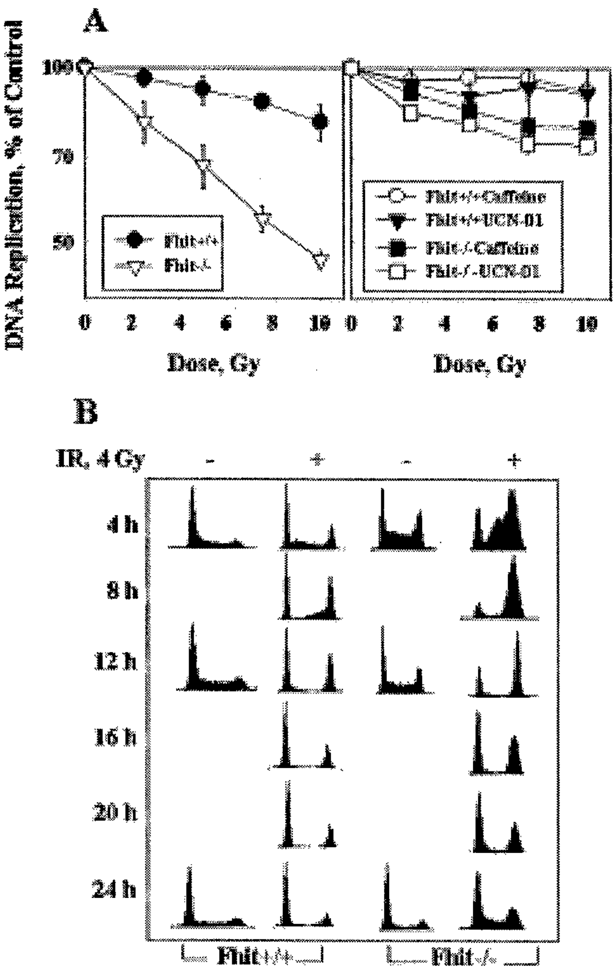

Fig. 1. **Stronger S and G2 checkpoint responses are shown in irradiated Fhit-/- cells. A:** DNA replication of Fhit cells following IR. ^{14}C-Tdr pre-labeled cells received pre-warmed medium with or without treatment of caffeine (4 mM) or UCN-01 (100 nM) for 30 min before IR. At 3 h after exposure to different doses of IR, 0.5 μM ^{3}H-Tdr was added to the cell cultures. Thirty min later the cells were collected and loaded on GF-A filters set in a Millipore Vacuum chamber. The following procedures are as described previously (6). The rate of DNA synthesis for each sample was calculated as ^{3}H dpm/^{14}C dpm and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time-point. The data are presented as mean values and standard deviations from three independent experiments. **B:** G2 arrest in Fhit cells following IR. The Fhit cells were collected at indicated time-points after exposure to 4 Gy and fixed in 70% ethanol. Cells were washed with PBS and stained with the solution (62 μg/ml RNase A, 40 μg/ml propidium iodide and 0.1% Triton X-100 in phosphate-buffered saline) at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite).

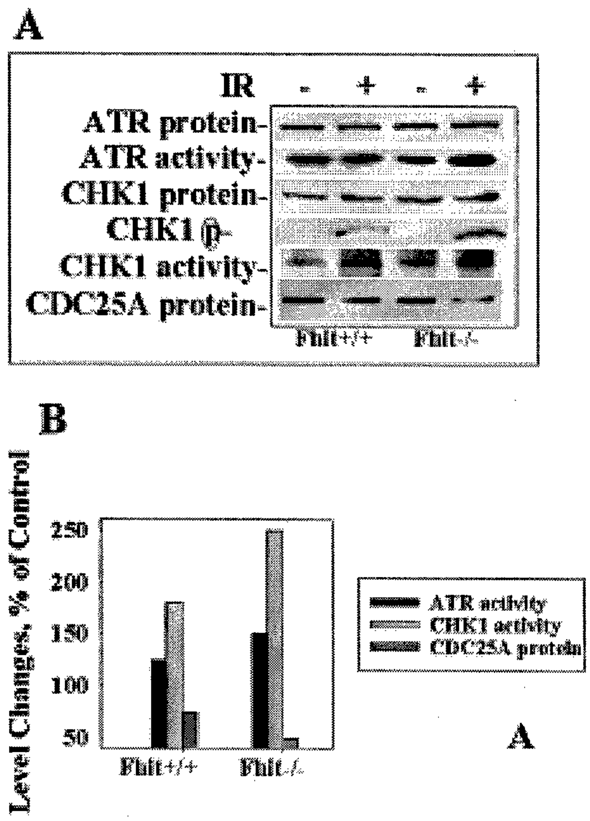

Fig. 2. **An over-activation of the ATR/CHK1 pathway is shown in irradiated Fhit$^{-/-}$ cells. A:** The Fhit cells were collected 6 h after IR (10 Gy). Protein levels were measured by using nuclear extracts from non-or irradiated Fhit cells. The kinase activities were measured in ATR or CHK1-immunoprecipitated nuclear extracts according to the protocols described in "Materials and Methods". **B:** Activity levels were obtained by quantifying ATR and CHK1 activities, as well as CDC25A levels shown in (A), using the PhosphoImager software. Similar results were obtained from two independent experiments.

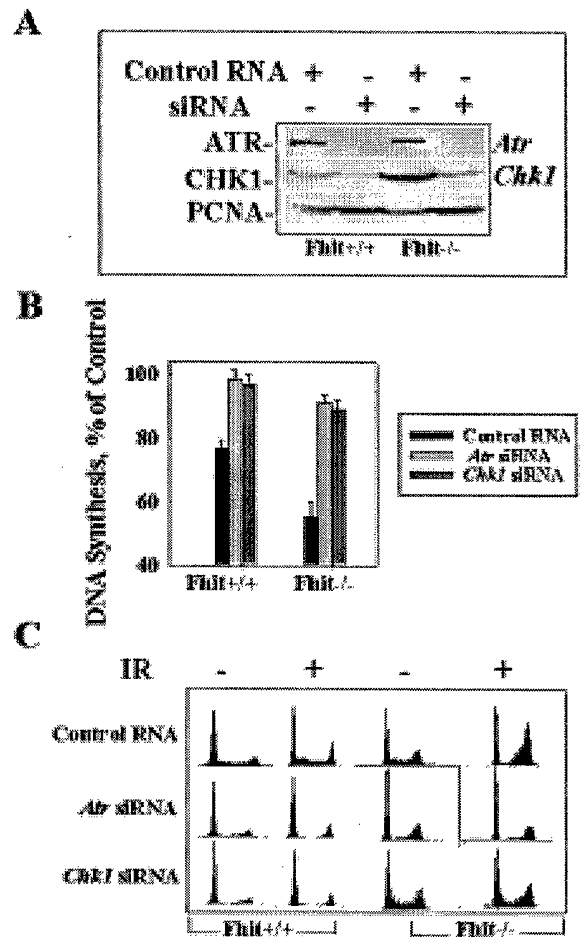

Fig. 3. ***Atr* or *Chk1* siRNA abolishes the stronger S and G2 checkpoint responses in irradiated Fhit$^{-/-}$ cells. A:** The levels of ATR and CHK1 expression were measured with the extracts from either siRNA of *Atr* and *Chk1* antisense or control RNA treated Fhit cells. PCNA was used as the internal control. **B:** DNA replication measured in *Atr* or *Chk1* siRNA treated cells following IR. The treatments of *Atr* or *Chk1* siRNA are as described in "Materials and methods". The Fhit cells were collected at 3 h after 4 Gy of exposure. The ratios of DNA replication rates are presented as mean values and standard deviations from three independent experiments. **C:** G2 arrests measured in *Atr* or *Chk1* siRNA treated cells following IR. As described in "Materials and methods", Fhit cells were treated with siRNA of *Atr* or *Chk1* for 36 h and then irradiated (2 Gy). At 6 h after IR, cells were collected for preparation and measurement of flow cytometric profiles of cell cycle distribution as described in Figure 1.

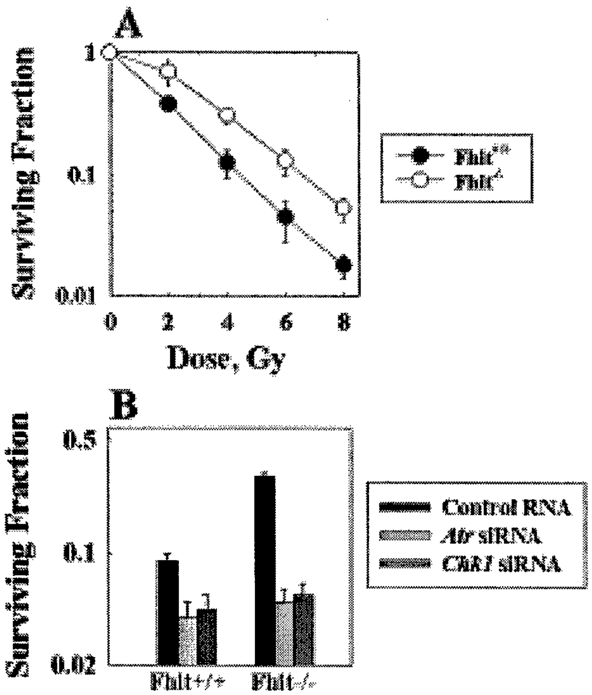

Fig. 4. **The radioresistance of Fhit$^{-/-}$ cells is diminished by *Atr* or *Chk1* siRNA treatment. A:** Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (7). Data shown are the average from three independent experiments. **B**: *Atr* or *Chk1* siRNA sensitized Fhit cells to IR-induced killing. At 36 h after siRNAs treatment cells were exposed to 4 Gy, then trypsinized and planted in medium without oligonucleotide. Data shown are the average from three independent experiments.

KEY RESEARCH ACCOMPLISHMENTS:

We reported that Fhit$^{-/-}$ cells are more resistant to IR-induced killing than Fhit$^{+/+}$ cells. The radioresistant phenotype of Fhit$^{-/-}$ cells is associated with stronger S and G2 checkpoint responses cells regulated by an over-activated ATR pathway (4). In addition, we reported that ATR protects cell from IR-induced killing is linked to HRR but not NHEJ (5).

REPORTABLE OUTCOMS:

Manuscripts:

1. Hu, B., Han, S-Y., Wang, X., Ottey, M., Potoczek, M.B., Dicker, A., Huebner, K., and **Wang, Y.** Involvement of the *Fhit* gene in the ionizing radiation-activated ATR/CHK1 pathway. J. Cell Physiol. DOI 10.1002/jcp.20222, 2004.
2. Wang, H.Y., Wang, H.C., Powell, S.N., Iliakis, G. and **Wang, Y.** ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of non-homologous end joining. Cancer Res. 64: 7139-7143, 2004.

Developed siRNAs:

1. ATR (4)
2. Chk1 (4)

Funding applied for based on work supported by this award:

One application entitled "Characterization of the role of Fhit in maintenance of genomic integrity following low dose radiation, *in vivo* and *in vitro*" is in response to the Department of Energy, Office of Science Notice DE-FG01-04ER04-21: Low Dose Radiation Research Program-Molecular Mechanism and Pathways, and addresses research to elucidate the mechanism by which Fhit maintains genomic integrity by affecting checkpoint and DNA repair in *vitro* and *in vivo*, following low dose ionizing radiation (IR).

CONCLUSIONS:

These findings provide an explanation for the relationship between Fhit expression and breast cancer progression. The importance of this research is that it will provide theory guidance for improving clinical treatment of breast cancers in which Fhit is deleted or reduced by combining traditional therapy with blocking the ATR pathway.

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4. **Hu B, Han S-Y, Wang X, et al. Involvement of the Fhit gene in the ionizing radiation-activated ATR/CHK1 pathway. J Cell Phys 2004;DOI 10.1002/jcp.20139.**
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